Purified Glycosaminoglycans from Cooked Haddock May Enhance Fe Uptake Via Endocytosis in a Caco-2 Cell Culture Model

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ABSTRACT: This study aims to understand the enhancing effect of glycosaminoglycans (GAGs), such as chondroitin/dermatan structures, on Fe uptake to Caco-2 cells. High-sulfated GAGs were selectively purified from cooked haddock. An *in vitro* digestion/Caco-2 cell culture model was used to evaluate Fe uptake (cell ferritin formation) from a Fe^{+3}-containing solution, and Fe^{+3}/ascorbic acid (AA) and Fe^{+3}/GAGs mixtures. Mitochondria (MTT test) and endosomal/lysosomal activities (neutral red uptake, NR), intracellular accumulation of reactive oxygen species, and GSH concentration were monitored as biomarkers of the changes of cellular metabolism. Changes in mRNA expression of Fe transporters, divalent metal transporter-1 (DMT1), and duodenal cytochrome-b (DcytB) were also evaluated. The Fe uptake from Fe^{+3}/GAGs mixture was up to 1.8-fold higher than from Fe^{+3} alone. Both Fe^{+3} alone and Fe^{+3}/AA mixture produced highest increase in MTT conversion. In contrast, cell cultures exposed to the Fe^{+3}/GAGs mixture exhibited highest NR uptake values. All Fe-containing solutions tested caused a sharp intramitochondrial accumulation of reactive oxygen species. Cell cultures exposed to the Fe^{+3}/GAGs mixture exhibited a more preserved (by 8%) intracellular GSH concentration compared to cultures exposed to Fe^{+3} or Fe^{+3}/AA mixture. In addition to cell responses, the mRNA expression of Fe transporters may suggest that Fe could also be internalized into cells by endocytosis in addition to via DMT1 in Fe^{+3}/GAGs mixtures. These aspects need to be confirmed in *in vivo* experiments to better establish nutritional interventional strategies.

Keywords: Caco-2 cells, cell metabolism, Fe uptake, glycosaminoglycans, oxidative stress

Introduction

Iron (Fe) deficiency is a common and devastating nutritional disorder in the world (Lewis 2005), estimated affecting 20% to 50% of the world's population (Beard and Stoltzfus 2001). Among other factors, poor bioavailability of Fe in the diet and the lack of highly available forms of Fe such as heme-Fe (present in meat) constitute key causes of Fe deficiency. Human studies are time-consuming, expensive, and have limited capacity to address interactions of Fe within foods in the gut. This fact has led to develop *in vitro* methods to evaluate Fe bioavailability such as Caco-2 cell culture, which has been proved to predict the correct direction, but not the magnitude, of inhibitors and enhancers on Fe uptake (Fairweather-Tait and others 2005).

Muscle tissue (meat) consumption enhances nonheme-Fe absorption, and in such effect the participation of both protein and no-protein components have been proposed (Hurrell and others 2006). However, only *in vitro* studies have shown that acidic carbohydrates present in the extra cellular matrix, glycosaminoglycans (GAGs), from fish muscle promote Fe uptake to Caco-2 cells (Huh and others 2004; Laparra and others 2008a). The GAGs are structurally diverse and can be present in several biological forms; 7 different structures for GAGs have been described: hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate and heparins, and keratan sulfate (Sasisekharan and Myette 2003). It should be pointed out that in muscle tissue the GAGs are covalently linked to a protein core forming proteoglycans (Gallagher 1989; Tingbø and others 2005). Presently, there are scarce studies, which evaluated the GAGs composition in fish muscle (Laparra and others 2008a; Tingbø and others 2005; Hannesson and others 2007). Tingbø and others (2005) reported a different proportion of chondroitin/dermatan-sulfate (CS/DS) in wolfish (58%) than cod (25%), and keratan sulfate (KS) constituted the lowest proportions (3%). In a 1st attempt to evaluate the proportion of GAGs in cooked haddock, the high-sulfated fraction was estimated to constitute a 0.56% relative to the initial fish muscle weight (Laparra and others 2008a). Interestingly, Hannesson and others (2007) reported a different composition of CS- and KS-proteoglycans between cod and bovine muscle.

It is accepted that absorption of dietary Fe takes place by the coordinated action of DcytB, a Fe-regulated ferric reductase with a heme containing b-type cytochrome associated with DMT1 (Divalent metal transporter-1) both located in the brush border membrane of enterocytes (McKie and others 2001). The intracellular pool of Fe regulates both the absorption of Fe at intestinal level and the iron regulatory proteins (IRP) (Arredondo and others 1997). It has been reported that IRP1 registers cytosolic Fe and oxidative stress through its labile cytosolic pool and the mitochondria iron–sulfur (Fe–S) clusters (Pantopoulos 2004; Tong and Rouault 2007). Although, Fe is required for numerous cellular functions it has the capacity to generate oxidative stress, which after long term can cause misregulation of Fe homeostasis and subsequently contribute to different diseases (Pietrangelo 2003; Napier and others

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doi: 10.1111/j.1750-3841.2009.01216.x

THE Society for Food Science & Technology
Journal of Food Science
A Publication of the Institute of Food Technologists

2005). Interestingly, an *in vitro* antioxidant ability for GAGs in skin fibroblast cultures has been suggested (Campo and others 2005), probably due to their capacity to chelate transition metals such as Fe^{+2} (Campo and others 2004). It is known that GAGs are internalized into cells by endocytosis processes (Yanagishita and Hascall 1992); however, to the best of our knowledge there are no previous studies evaluating the effect(s) of dietary GAGs on cell responses in a cell culture model of intestinal epithelia.

Our group has been focused on GAGs since first we observed that an acid extract from cooked haddock, without need of enzymatic digestion, contributed to the promoting effect of fish muscle on Fe uptake (Huh and others 2004). In the latter study, the promoting fraction resulted rich in carbohydrates, and a preliminary analysis via ^{1}H NMR suggested that heparin-like-GAGs were the major constituents. In a recent study, we applied a selective purification process for GAGs according to their sulfation pattern and further confirmed the promoting effect of chondroitin/dermatan sulfate related compounds on Fe uptake to Caco-2 cells (Laparra and others 2008a). The present study continues our previous research and aims to monitor the cellular responses of a human-derived intestinal cell line (Caco-2) to Fe uptake in presence of purified sulfated GAGs. These data would allow us to gain insights about the potential biochemical system(s) involved on Fe uptake, which could be affected by GAGs. These aspects are of interest because Fe homeostasis in mammals is maintained through the tight regulation of its intestinal absorption.

Materials and Methods

Reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All glassware used in the sample preparation and analyses was treated with 10% (v/v) of HCl concentrated (37%) for 24 h, and then rinsed with deionized water (18 MΩ cm) before being used.

Instruments

Fe concentration was determined with an inductively coupled argon plasma emission spectrometer (ICP-ES, Model 61E Trace Analyzer, Thermo Jarrell Ash Corp., Franklin, Mass., U.S.A.) after wet-ashing (Laparra and others 2008b). Other equipment used included a spectrophotometer (DU 520 UV/vis, Beckman Coulter, Palo Alto, Calif., U.S.A.), and an automatic gamma counter Wizard 3 Wallac 1480 (Perkin Elmer, Norwalk, Conn., U.S.A.).

Preparation of fish

Fresh samples of haddock were purchased from local markets of Ithaca (N.Y., U.S.A.) and all visible fat and skin were removed. The fillets (100 g) were cut into small pieces, mixed with 55 mL of deionized water, and then cooked in a domestic microoven (1000 W) for 1.5 min, stirred, and cooked for an additional 1.5 min. The cooked slurry was poured into ice cube trays and frozen at −20 °C. The frozen slurry was lyophilized and ground; the resulting powder was stored at −20 °C until use.

Purification of GAGs

Isolation of the high-sulfated fraction of GAGs from cooked haddock was performed as previously described (Laparra and others 2008a). Briefly, aliquots (5 g) of the lyophilized cooked fish were digested with papain (Cat. Nr 76216, Sigma Chemical Co.) (4 μg/mg of tissue) in 10 volumes of 0.1 M sodium phosphate buffer, containing 0.005 M EDTA, 0.005 M cysteine hydrochloride, and 0.02% (w/v) sodium azide, at 65 °C for 24 h. After protein precipitation (by adding trichloroacetic acid 7%, w/w), the supernatants were dialyzed (15,000 Da) in distilled water for 24 h. Then cetylpyridinium chloride was added drop wise (final concentration of 5 mM) to the retentates. The precipitate was sequentially washed with 5 mL 0.4 M and 2.1 M NaCl to isolate the GAGs according their low- and high-sulfur content. To the 2.1 M NaCl fraction was added potassium thiocyanate in a molar ratio (1:1) with cetylpyridinium, and the resulting precipitate was removed by filtration (Whatman N° 41). The filtrate was dialyzed in deionized water for 24 h, and the retentates were freeze-dried before use.

Cell cultures

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.) at passage 17 and used in experiments at passage 25 to 33. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described (Laparra and others 2008c).

The cells were used for Fe uptake experiments at 13-d post-seeding. For the assays, Caco-2 cells were seeded at 50000 cell cm^{-2} in collagen-treated 6-well culture plates (Costar, Cambridge, Mass., U.S.A.), and were grown with DMEM. On the day prior to the *in vitro* digestion experiment, the DMEM medium was replaced by 2 mL of minimal essential medium (MEM, Gibco), and then the cells were returned to the incubator. Baseline cell ferritin in cultures grown in MEM averaged 2.1 ng/mg cell protein.

Samples

Cell cultures were exposed to *in vitro* digests of (1) $FeCl_3$ (41.7 μmol/L); (2) a $FeCl_3$ (41.7 μmol/L)/ascorbic acid (AA) mixture (molar ratio for Fe/AA, 1/20); (3) a $FeCl_3$ (41.7 μmol/L)/high-sulfated GAGs from fish (500 μg) mixture.

In vitro digestion

To simulate gastrointestinal digestion, a previously described method by Glahn and others (1998) was applied. Porcine pepsin (P-7000) (800 to 2500 units/mg protein), pancreatin (P1750) (activity, 4 × USP specifications) and bile extract (B8631) (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were de-mineralized with Chelex-100 (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) before use.

Peptic and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37 °C/5% CO_2/95% air relative humidity). After gastric digestion (pH 2), the intestinal digestion (pH 6.9 to 7) was carried out in the upper chamber of a 2-chamber system in 6-well plates. In the bottom chamber, 1 mL of MEM was placed. Then an aliquot (1.5 mL) of the gastrointestinal digest was loaded into the upper chamber and incubated for 2 h at 37 °C with gentle rocking. Afterwards, the inserts were retired and an additional 1 mL of MEM was added. The cell cultures were incubated for 22 h at 37 °C. Ferritin formation was used as an index of the cellular Fe uptake.

Ferritin analysis

The ferritin concentration and total protein concentration were determined on aliquots of the harvested cell suspension with a 1-stage sandwich immunoradiometric assay (FERIRON II Ferritin Assay, Ramco Laboratories, Houston, Tex., U.S.A.) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, Calif., U.S.A.), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular Fe concentration (Glahn and others 1998).

Mitochondria enzyme activities

These activities were evaluated by monitoring MTT (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide) conversion on exposed cultures after an incubation period (Laparra and others 2008b). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondria dehydrogenases yielding a blue formazan product, which can be measured spectrophotometrically. Control cells exposed to digests containing enzymes but not Fe were used throughout each assay.

Lysosomal activity

This activity was investigated by using the neutral red (toluylene red; 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay (Borenfreund and Puerner 1985). The medium was removed and cells were washed twice with PBS. The uptake of toluylene red was measured using a commercial kit (Sigma, nr 7H092), and absorbance was measured at 540 nm with background subtraction at 690 nm. Control cells exposed to digests containing enzymes but no Fe were used throughout each assay.

Intracellular reactive oxygen species (ROS) accumulation

The intracellular production of ROS in Caco-2 cells was measured using a 2 mM dihydrorhodamine 123 (DHR, Sigma) solution prepared in dimethylsulfoxide. The ROS production was determined according to Laparra and others (2008d). Cell cultures were analyzed by flow cytometry (Coulter, EPICS XL-MCL). Fluorescence was analyzed at $\lambda_{exc} = 488$ nm and $\lambda_{em} = 525$ nm. Control cells exposed to digests containing enzymes but no Fe were used throughout each assay.

Thiol–redox balance

For evaluating the thiol-redox balance, the incubated (2 h) cell cultures were treated with 0.5 mL of 20 mM Tris buffer solution containing 0.1% Triton (v/v) to obtain the cell homogenate. The redox balance in Caco-2 cultures was determined as previously described (Laparra and others 2006). The concentrations of GSH were normalized by determination of total protein content using a Bio-Rad Protein Assay kit II (cat. nr 500-0002).

mRNA analysis of the Fe transporters

Cells were harvested and were immediately transferred to liquid nitrogen. Total RNA was isolated from cell cultures using a commercial kit (Rneasy Midi-Maxi kit, Qiagen, Calif., U.S.A.) and reverse-transcribed using oligo (dT) and Superscript II reverse transcriptase (MBI, fermentas).

First strand cDNAs were synthesized from 5 μg of total RNA from cell cultures using oligo $(dT)_{18}$ as primers in the presence of MLV reverse transcriptase (Fermentase), for 1 h at 42 °C. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as previously described (Laparra and others 2008b).

Statistical analysis

A 1-factor analysis of variance (ANOVA) and the Tukey test were applied to determine differences between treated and control cultures (Box and others 1978). The experiments were performed in triplicate in 2 different days ($n = 6$). A significance level of $P < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.0 (Rockville, Mass., U.S.A.) was used for the statistical analysis.

Results and Discussion

Caco-2 cell model was shown to have high sensitivity to available Fe, and exhibited a maximal absorption capacity when exposed to Fe concentrations between 20 and 50 μM (Glahn and others 1998). In the present study, the AA proportion (Glahn and others 1998) and Fe concentration used (41.7 μmol/L) (Huh and others 2004) was chosen from previous of our studies. Cooked haddock was used due to its low concentration of intrinsic Fe (6.7 ± 0.5 μg as Fe/g muscle tissue, dry basis), and allow us to refer the potential effects of GAGs noted over the extrinsic Fe added in the mixtures. According to our previous data the ICP-ES analysis of the purified fraction of GAGs revealed the nondetectable Fe concentration in this extract. The ratio Fe/GAGs used was established elsewhere, which is achievable through a daily consumption of a typical serving of meat (approximately 150 g/d) (Laparra and others 2008a). The nature of the main compounds in the purified fraction of GAGs was approached through reverse phase HPLC analysis, which pointed to chondroitin/dermatan sulfate structures (Laparra and others 2008a). Furthermore, these results were confirmed by an enzymatic assay, where chondroitinase ABC was shown to selectively digest the main peak of the purified fraction.

Fe uptake and cell metabolic responses

Ferritin concentrations in cultures exposed to digests of the Fe^{+3} and Fe^{+3}/AA solution, and Fe^{+3}/GAGs mixture are shown in Figure 1. Ferritin concentrations in cell cultures exposed to digests of Fe^{+3}/GAGs were 1.8-fold higher compared to that quantified in cultures exposed to Fe^{+3}-containing solution. These results evidence the promoting effect of purified GAGs on Fe uptake to Caco-2 cells, and confirm one more time those reported by our group when using similar experimental conditions (Huh and others 2004; Laparra and others 2008a). However, it is worthy to point out that this effect has only been evidenced *in vitro*. A recent *in vivo* study reported that nonheme-Fe absorption in young women is not influenced by some commercially available GAGs such as sodium hyaluronate and chondroitin sulfate (from shark cartilage) when added in a 0.1% (w/w) relative to the total amount of diet (Storcksdieck and others 2007). The different reasons, which can be responsible for and explain the differences between results from our *in vitro* experiments and the *in vivo* study lay on structural differences of the GAGs tested and the dose, which is achievable through dietary consumption of meat (Laparra and others 2008a).

Cell metabolic responses in mitochondria and lysosomal/endosomal activities are shown in Figure 2. When considering

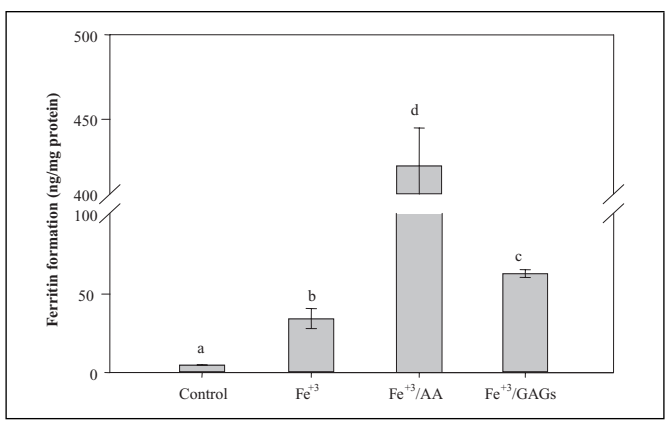

Figure 1—Ferritin levels in Caco-2 cells exposed to a $FeCl_3$ standard solution, and jointly with GAGs (500 μg *in vitro* method) or ascorbic acid (1:20 molar ratio). Samples were prepared with $FeCl_3$ (final concentration 41.7 μmol/L as Fe) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation ($n = 6$). Different letters indicate statistical ($P < 0.05$) differences.

changes in mitochondria enzyme activities, a statistically ($P < 0.05$) significant increase in MTT conversion was observed in cultures exposed to all of the samples tested compared to the controls. There was no significant ($P > 0.05$) difference in MTT conversion values in cultures exposed to digests of Fe^{+3} solutions or the Fe^{+3}/GAGs mixtures. Pilot studies in our laboratory demonstrated that GAGs did not affect MTT conversion percentages, but only increased significantly the neural red uptake percentages. In cell cultures challenged to the Fe^{+3}/AA mixtures the highest MTT conversion values were noted. Regarding neutral red uptake, there were no significant ($P > 0.05$) differences in neutral red uptake percentages in cultures exposed to digests of Fe^{+3}-containing solutions compared to the controls. Otherwise, when highly available Fe from Fe^{+3}/AA and Fe^{+3}/GAGs mixtures was exposed to cell cultures higher neutral red uptake percentages were quantified.

Mitochondria enzyme activities were proved as sensitive biomarkers of changes in cellular metabolism in response to Fe uptake (McKie and others 2001; Laparra and others 2008a), being linked to DcytB activity (McKie and others 2001). In addition, cellular metabolic responses noted suggest a stimulated metabolism in the lysosomal/endosomal compartment in the presence of GAGs and AA. The fact that once GAGs reach the cell surface they are mainly internalized into cells by endocytosis and degraded in the acidic compartments of the lysosomal/endosomal systems (Yanagishita and Hascall 1992) would explain the increased neutral red uptake noted in cultures exposed to the Fe^{+3}/GAGs mixture. In addition, a rapid (few hours and maximum in 4 h) DMT1 mobilization from the cell brush border membrane to a late lysosomal/endosomal compartment when Fe^{+2} (100 μM) was presented to the apical surface of Caco-2 cells has been reported (Johnson and others 2005). The latter observation may explain the increased neutral red uptake noted in cultures exposed to the Fe^{+3}/AA solution.

Presently, it is assumed that nonheme-Fe enters the body across the apical membrane of enterocytes via DMT1 (Sharp and others 2002; Johnson and others 2005), a pH-dependent and Fe^{+2}-specific (McKie and others 2001) way. Once internalized into cells the intracellular pool of free Fe regulates the iron regulatory proteins (IRP) activities (Arredondo and others 1997). Therefore, it was of interest to relate the variations of the cellular metabolic responses to the expression of the described transporters for Fe at the intestinal level.

mRNA expression of Fe transporters

Caco-2 relative DMT1 and DcytB mRNA expression changes are shown in Figure 3. mRNA expression analysis revealed that DMT1 was downregulated ($P < 0.05$) in cultures exposed to the Fe^{+3}/AA mixture, where Fe is highly available. There were no significant ($P > 0.05$) differences in DMT1 mRNA expression changes in cultures exposed to digests of either Fe^{+3} solution or Fe^{+3}/GAGs mixture when compared to the controls. Meanwhile, the PCR analysis revealed that DcytB mRNA expression was upregulated in cultures exposed to digests of both Fe^{+3} solution and Fe^{+3}/GAGs mixture relative to the controls. The exposure to Fe^{+3}/AA mixture caused no change in DcytB mRNA expression compared to the controls.

DMT1 was quickly (after 2 h) downregulated in cell cultures exposed to Fe^{+3}/AA mixture. This observation is in accordance with our previous study where Caco-2 cultures were exposed to a highly soluble Fe supplement (FeEDTA), but in lower concentration (12 μM), showed downregulated DMT1 mRNA expression (Laparra and others 2008b). It is known that intestinal Fe absorption is affected by several different dietary factors, but despite the obvious importance of DMT1 in maintaining Fe homeostasis there is no information concerning the regulation of the DMT1/DcytB transport pathway in intestinal cells by dietary factors that affect Fe bioavailability such as AA and GAGs.

DcytB has been described as a ferric reductase, with a heme containing b-type cytochrome, associated with DMT1 in the absorption of dietary Fe (McKie and others 2001). It is accepted that dietary Fe^{+3} is reduced by DcytB and transported into cells by DMT1. The fact that AA promotes Fe uptake by reducing Fe^{+3} (Hallberg and others 1989) can explain the nonaltered DcytB mRNA expression in cell cultures challenged to digests of the Fe^{+3}/AA mixture relative to the controls. On the other hand, when no reducing agent was present in the media cell cultures exposed to digests of the Fe^{+3} solution exhibited an upregulation on DcytB mRNA expression. A recent study provided interesting suggestions

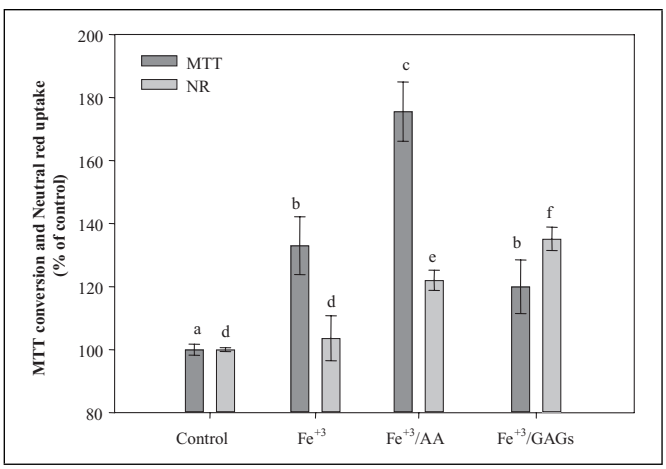

Figure 2—Mitochondrial (MTT test) and lysosomal (Neutral red, NR, uptake) activities in Caco-2 cells exposed to a $FeCl_3$ standard solution, and jointly with GAGs (500 μg *in vitro* method) or ascorbic acid (1:20 molar ratio). Samples were prepared with $FeCl_3$ (final concentration 41.7 μmol/L as Fe) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation ($n = 6$). Different letters for each assay (dark bars compared to dark bars and light gray bars compared to light gray bars) indicate statistical ($P < 0.05$) differences.

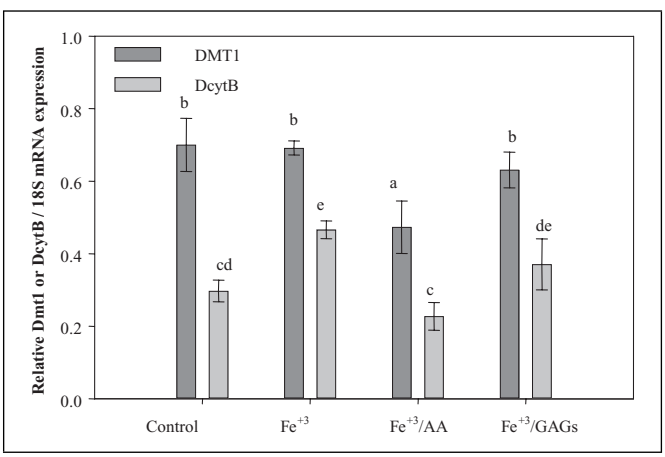

Figure 3—Divalent metal transporter (DMT1) and duodenal ferrireductase (DcytB) relative expression in Caco-2 cells exposed to a $FeCl_3$ standard solution, and jointly with GAGs (500 μg *in vitro* method) or ascorbic acid (1:20 molar ratio). Samples were prepared with $FeCl_3$ (final concentration 41.7 μmol/L as Fe) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation ($n = 6$). Different letters for each assay (dark bars compared to dark bars and light gray bars compared to light gray bars) indicate statistical ($P < 0.05$) differences.

of the metal chelating ability for GAGs, which would be due to their particular chemical structure allowing the interaction of charged groups, such as uronic acid and/or the carboxylic group of hexosamine, with transition metals (Campo and others 2004). This suggested metal chelating ability would explain the nonsignificant ($P > 0.05$) differences of DcytB mRNA expression in cultures exposed to digests of Fe^{+3}/GAGs mixtures relative to the controls.

In the present study, there were no significant ($P > 0.05$) differences in mRNA expression of both Fe transporters in cultures exposed to digests of the Fe^{+3} solution and Fe^{+3}/GAGs mixture. Thus, we cannot rule out that part of the Fe^{+3} would be able to interact with the DcytB/DMT1 transport system. Accordingly, reducing equivalents are needed which would explain the nonstatistical ($P > 0.05$) differences in MTT conversion percentages in cultures exposed to digests of the Fe^{+3} solution and Fe^{+3}/GAGs mixture. However, higher Fe uptake values were quantified in cultures exposed to digests of the Fe^{+3}/GAGs mixtures (Figure 1). From this scenario, it seems that GAGs would promote Fe uptake to Caco-2 cells by stimulating the endocytosis processes which may help to internalize Fe into cells. This observation would be relevant since mammals maintain the Fe homeostasis mainly through a tight regulation of the intestinal Fe absorption. From a physiological point of view, the suggested involvement of GAGs-induced endocytosis processes on Fe uptake would allow higher Fe uptake amount without trafficking away DMT1 from the brush border membrane. The latter may explain the higher ferritin formation values in cultures exposed to digests of the Fe^{+3}/GAGs mixture without significant differences in Fe transporters expression patterns. In addition, Fe uptake through GAGs-induced endocytosis would prevent the mitochondria Fe overload in Fe-deficient cultures re-exposed to Fe-containing solutions (Yang and others 2006). These aspects are discussed in the next section.

Cell redox status

It is accepted that Fe is an essential micronutrient, but excess of free Fe has the capacity to generate oxidative stress to Fe-deficient cells (Pietrangelo 2003; Napier and others 2005). To gain insights of the oxidative status in cell cultures exposed to digests of Fe^{+3}, Fe^{+3}/AA, and Fe^{+3}/GAGs mixture, the intramitochondrial production of reactive oxygen species (ROS) and GSH concentrations, as the main antioxidant defense system to metabolize ROS in Caco-2 cells (Baker and Baker 1993), were evaluated (Figure 4). In all of the assayed cases, compared to the controls, an increase in cellular ROS accumulation was observed. As observed, differences on Fe availability in Fe^{+3} and Fe^{+3}/AA solutions did not cause significant differences on ROS production. Nevertheless, a statistically significant ($P < 0.05$) reduction of ROS level by 8% was noted in cell cultures exposed to Fe^{+3}/GAGs mixture.

Under the experimental conditions used in the present study Caco-2 cultures are Fe deficient (Glahn and others 1998), the ferritin levels quantified in control cultures support our statement (Figure 1). It has been reported that in Fe-deficient conditions cells respond by disassembling the Fe–S cluster in IRPs, which result converted in apo-IRPs (Pantopoulos and Hentze 1995). In the mitochondria, Fe–S cluster biogenesis is needed not only to support respiratory functions and heme biosynthesis, but also for sensing and regulation of mitochondria homeostasis (Rouault and Tong 2005; Pierrel and others 2007). Afterwards, when Fe is available the previously disrupted synthesis of the Fe–S cluster cause the mitochondria Fe overload (Yang and others 2006). Reactive oxygen intermediates (that is, superoxide anion and hydrogen peroxide), are products of aerobic metabolism. This cascade of events would

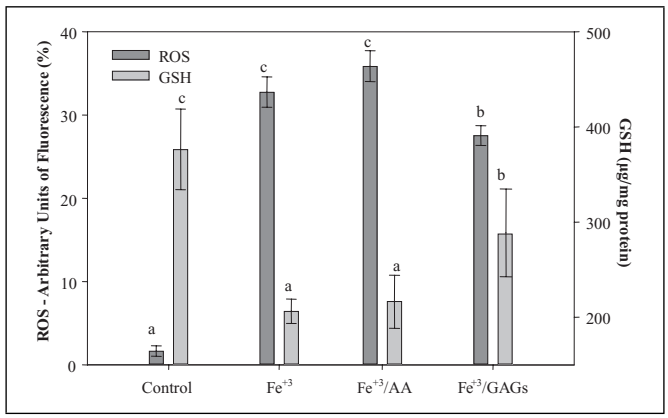

Figure 4—Intramitochondrial reactive oxygen species (ROS) and glutathione (GSH) concentrations in Caco-2 cells exposed to a $FeCl_3$ standard solution, and jointly with GAGs (500 μg *in vitro* method) or ascorbic acid (1:20 molar ratio). Samples were prepared with $FeCl_3$ (final concentration 41.7 μmol/L as Fe) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation ($n = 6$). Different letters for each assay (dark bars compared to dark bars and light gray bars compared to light gray bars) indicate statistical ($P < 0.05$) differences.

explain the increased and close values for intramitochondrial ROS accumulation noted in all cultures. The close ROS levels in all cases would be reflecting the homeostasis of Fe within the mitochondria (Tong and Rouault 2007).

Caco-2 cells exhibit different antioxidant systems (Wijeratne and others 2006), the GSH cycle being the most important to metabolize ROS (Baker and Baker 1993). In all assayed cases, the GSH depletion in cell cultures reflects the alteration in cell redox status, in agreement with the increased ROS levels detected (Figure 4). Both ROS (Shackelford and others 2000) and GSH precursors (Nkabyo and others 2002) can alter cell populations in cycle phases. However, cell cycle studies conducted on cultures exposed to all the conditions tested revealed no significant alteration in cycle progression (data not shown). Cell cultures exposed either to Fe^{+3} alone or Fe^{+3}/AA mixture showed similar ($P > 0.05$) GSH concentrations (Figure 4), which resulted statistically ($P < 0.05$) lower than in control cultures. The GSH concentration in cultures exposed to Fe^{+3}/GAGs was also statistically ($P < 0.05$) decreased relative to the controls; however, it appears that GSH was slightly preserved. The reduced pool of free Fe in the Fe^{+3}/GAGs mixture would explain the more preserved cell redox status. The latter hypothesis would be in agreement with our speculation that GAGs stimulate endocytosis. The potential antioxidant effect for GAGs is concordant with the decreased $OH^{\bullet}$ generation and reserved intracellular GSH concentrations caused by purified human GAGs (0.5 to 2 mg/mL) in skin fibroblast cultures exposed to $FeSO_4$ (2 μM) plus ascorbic acid (1 mM) (Campo and others 2005).

Conclusions

In summary, the cellular responses noted in cell cultures exposed to Fe^{+3}/GAGs mixture may suggest that Fe would also be internalized into cells through a different pathway, probably GAGs-induced endocytosis processes, in addition to that generally accepted via DMT1 in the enterocytes. The fact that GAGs are metabolized in the acidic compartments of the endosomal/lysosomal systems (Yanagishita and Hascall 1992) would support this hypothesis. The lowered pool of free Fe in the cellular cytosolic compartment could contribute to reduce the oxidative status in Fe-deficient cells re-exposed to available Fe caused by the mitochondria overload. The latter observation would help to store Fe into ferritin.

The data concerning cellular response(s) for main Fe transporters described until now might suggest that both internalization routes, via DMT1 or endocytosis, would participate on Fe uptake. However, further studies are needed to confirm *in vivo* the enhancing effect of GAGs and the potential participation of endocytosis processes on Fe uptake.

Acknowledgments

This study was supported by the USDA Robert W. Holley Center for Agricultural Research and the Dept. of Food Science at Cornell Univ. Dr. José Moisés Laparra was sponsored as a Fulbright Scholar and supported by a postdoctoral fellowship from the Spanish government.

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